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# Azospirillum

**IV** Genetics · Physiology · Ecology

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**AZOSPIRILLUM SPP ECOLOGY**  
**OF SOME SOILS OF THE SOMALI REPUBLIC**

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Summary: During a survey of the distribution of nitrogen-fixing bacteria in soil samples (12 alluvial and 1 sandy) collected in two localities (Jenale and Afgoj) in the Somali Republic, microorganisms resembling Azospirillum were isolated and characterized.

All the soil samples exhibited weak nitrogenase activity when incubated in presence only of sodium malate. Azospirillum-like bacteria occurred only in the 12 alluvial soil samples and not in the sandy one.

From the 4 soil samples, with the highest nitrogenase activity 18 Azospirillum strains were isolated altogether, and these were preliminarily identified as: Azospirillum brasilense nir+ (13 strains), Azospirillum brasilense nir- (1 strains).

Four strains showing physiological characteristics of the species Azospirillum lipoferum did not fit completely the description of the species and were considered very close to Azospirillum lipoferum.

The Azospirilla detected in these soils represented a considerable part (30-40%) of the entire diazotrophic population; in which Azotobacter sp., Beijerinckia sp. and heterocistous cyanobacteria (Nostoc sp., Calothrix sp., Anabaena sp. and Scytonema sp.) occurred.

The collected data also represent the first contribution to our

knowledge of the ecology of Azospirillum spp. in the soils of the Somali Republic.

Keywords: Azospirillum, occurrence, tropical soils.

## Introduction

Nitrogen-fixing bacteria of the genus Azospirillum have been reported to occur in soils and in association with the roots of several plants widely distributed in tropical and temperate regions (1-2 - 3-4).

Moreover, the high incidence of Azospirillum in soils and in root samples from tropical regions relative to those from temperate zones can be attributed either to the low level of available nitrogen in tropical soils or to the higher temperature requirement of these microorganisms (5-6-7).

In a survey of the distribution of nitrogen fixing bacteria in Somalian soils, microorganisms resembling Azospirillum were isolated and characterized.

## 1 Material and Methods

### 1.1 Samples

The soil samples (12 alluvial and 1 sandy, each uncultivated) were collected at the end of the dry season in 1985 in several localities in the districts of Jenale and Afgoj in the Somali Republic.

The collected soil samples were stored in polyethylene bags at 10°C for two weeks, then transported to the laboratory (in Italy), where they were examined for the occurrence of nitrogen-fixing microorganisms with particular attention to the presence

of Azospirillum.

### 1.2 Nitrogenase activity of the soils

Ten grams of each soil sample either without C source or with 1% of sodium malate were put into serum bottles (28 ml) and 1 ml of distilled water was added to each bottle.

The bottles were capped and preincubated for 16 h at 33°C in an atmosphere of  $N_2: O_2$  (99:1 v/v). After incubation the atmosphere was replaced with a mixture of air and acetylene (87.5:12.5 v/v) and the bottles incubated again for 1 h.

Ethylene was assayed by gas chromatography and nitrogenase activity was expressed as n.moles of  $C_2H_4$  produced per gram (dry weight) of soil per hour.

### 1.3 Isolation and characterization of Azospirillum from soil samples

Enrichment cultures of Azospirillum were prepared by introducing 0.1 g of soil to 20 ml test tubes containing 5 ml of N-free semisolid malate medium (8). After 48 h of incubation at 33°C the tubes showing white subsurface pellicle, nitrogenase activity more than 10 n.moles ethylene  $ml^{-1} h^{-1}$  as well the presence of very active bacteria with the typical spiral shape and poly-hydroxybutyrate (PHB) granules were considered as positive for the occurrence of Azospirillum-like forms.

Isolates of  $N_2$ -fixing spirilla were purified by several subcultures on Nfb supplemented with Congo Red according to the method described by Rodrigues-Caceres (9).

The identification of the isolates was made following the criteria suggested by Tarrand et al. (10), Baldani and Dobereiner (11) and Dobereiner (12).

Azospirillum lipoferum strain A3a from grass (Senegal), Azospirillum brasilense SP7 from Digitaria decumbens (Brasil) and Azospirillum brasilense strain cd from Cynodon dactylon (California) were used as reference strains.

The CFU of Azospirillum per gram (d.w.) of soil was estimated by the MPN method (13).

Nitrogen fixation of pure cultures of Azospirillum was tested by inoculating 0.2 ml of 48-h-old culture (grown in N-free semisolid medium) into 20 ml tubes with sodium malate and glucose as C sources.

The cultures were incubated for 48 h at 33°C. After incubation twelve per cent of the gas phase was replaced by acetylene and nitrogenase activity was expressed as n.moles of  $C_2H_4$  per milligram of protein per hour. The protein content of each culture was determined from three replicates by using a modified Lowry method (14)

## 2 Results and discussion

Each of the soil samples exhibited nitrogenase activity in the presence of 1% sodium malate though at different levels only (Tab.1).

In the majority of soil samples the levels of nitrogenase activity were between 18 and 76 n.moles  $C_2H_4$   $h^{-1} g^{-1}$  (d.w.) while in the samples AI, WBI, SH2 and SH5 the values were lower than 2 n.moles  $C_2H_4$   $h^{-1} g^{-1}$  (d.w.).

The enrichment cultures yielded Azospirillum-like forms only in the 12 alluvial soils and not in the sandy one (Table 2).

It was also possible to observe a direct correlation of the occurrence of Azospirillum-like forms with the nitrogenase

activity of the enrichment cultures and the number of azospirilla of the soil examined.

Table 1 - Nitrogenase activity and characteristics of the soil samples.

Samples	Localities	Soil	pH	n.moles $C_2H_4$ $g^{-1}$ (d.w.) $h^{-1}$ (a)
				(soil amended with 1% sodium malate)
WVI	OWDHEGLE (JENALE)	Alluvial	8.11	18.0
WV2	"	"	8.00	41.0
WV3	"	"	7.55	28.0
WV4	"	"	7.66	35.0
WBI	"	"	8.00	0.15
SH1	SHAAM (JENALE)	"	7.87	76.0
SH2	"	"	7.70	0.6
SH3	"	"	8.00	19.0
SH4	"	"	8.00	36.0
SH5	"	"	7.72	0.16
MI	MORDINLE (AFGOJ)	"	8.00	53.0
M2	"	"	8.01	18.0
AI	LAFOOLE (AFGOJ)	Sandy	7.51	1.6

(a) average of two experiments, each carried out with three replicates

The nitrogenase activity of the sample AI (sandy soil) can be ascribed to other microorganisms belonging to other  $N_2$ -fixing species. In fact the azospirilla detected in these soils represented a considerable proportion (30-40%) of the entire diazotrophic population, in which Azotobacter sp., Beijerinckia sp. (Favilli unpublished data) and heterocistous cyanobacteria (15) of the genus Nostoc sp., Calothrix sp., Anabaena sp. and

Scytonema sp. occurred as well. From four enrichment cultures showing higher levels of nitrogenase activity eighteen strains of Azospirillum were isolated altogether.

Table 2. Occurrence of Azospirillum-like forms, nitrogenase activity in the enrichment cultures and CFU of azospirilla in the soil examined.

Samples	Subsurface pellicle	<u>Azospirillum</u> -like forms (a)	$n$ , moles $C_2H_4$ $g^{-1}$ (d.w.) $h^{-1}$ (b)	CFU $10^3$ (d.w.) $g^{-1}$
WV1	+	+	38.74	420
WV2	+	++	61.00	560
WV3	+	+	48.65	450
WV4	+	++	55.40	540
WBI	+	+-	5.22	2.1
SH1	+	++	96.98	625
SH2	+	+-	6.95	1.00
SH3	+	+	39.66	250
SH4	+	+	56.77	15.0
SH5	+	+-	6.70	2.6
MI	+	++	73.97	570
M2	+	+	38.50	412
AI	-	-	6.37	-

(a) ++ elevated occurrence of Azospirillum-like forms

(b) average of two experiments, each with three replicates

Table 3 - Strain isolated from four soil sample enrichment cultures

Soil samples	n° of strain isolated	Isolate designation
WV2	6	1W; 2W; 3W; 4W; 5W; 6W;
WV4	2	7W; 8W
SH1	6	1S; 2S; 3S; 4S; 5S; 6S.
M1	4	1M; 2M; 3M; 4M.

Table 3 deals with the soil sample enrichment cultures from which the strains were isolated and the isolate designation.

## 2.1 Morphological, cultural and physiological characteristics of the strains isolated

After 24h in NFB semisolid malate medium the cells of the strains isolated from the four enrichment cultures were curved, highly motile rods with spiral movements. They contained PHB granules, and the size of the cells ranged from  $(0.9-1.4) \times (2.2-3.5) \mu\text{m}$ .

After 48h in alkaline NFB semisolid malate medium supplemented with  $\text{NH}_4\text{NO}_3$  (5mM) the cells of the strains 1W, 3W, 5W and 6W varied in length from 3.5 to 5.0  $\mu\text{m}$  without PHB granules.

On NFB malate agar ( $12.5 \text{ g l}^{-1}$ ) with yeast extract ( $0.1 \text{ g l}^{-1}$ ) the colonies were white, round and flat, becoming pink after 15 days.

After 24h on PSS agar (16) the colonies appeared white and creamy, becoming light red after 25-30 days.

On NFB semisolid malate medium all the strains developed a heavy white pellicle that became pink after 15-20 days.

The strains grew well on organic acids as malate and succinate while  $\alpha$ -Ketoglutaric acid, glucose, ribose and mannitol were utilized only by the strains 1W, 3W, 5W and 6W. The same strains utilized glucose, as C-source for nitrogenase activity with slight acidification of the medium (Table 4).

All the strains except strain 1S reduced nitrate to nitrite and nitrite to nitrous oxide or nitrogen gas.

All the strains were catalase positive and grew well without biotine.

Table 5 summarizes the morphological and physiological characteristics of the strains.

Table 4 - Nitrogenase activity of the strains during growth on malic acid and glucose as C sources.

48h-old culture on (NFB semisolid medium,  $pO_2$  0.001, pH 6.8)

STRAINS	n.moles $C_2H_4 h^{-1} g^{-1}$ protein <sup>(a)</sup>			
	malic acid		glucose	
		pH		pH
1W	39.34	7.7	13.79	6.4
2W	6.66	8.0	NG	-
3W	22.15	7.8	6.76	6.5
4W	7.76	7.8	5.98	6.3
5W	16.05	7.7	5.20	6.3
6W	71.67	7.8	NG(°)	-
7W	9.65	7.8	NG	-
8W	48.13	7.8	NG	-
1S	64.82	7.8	NG	-
2S	69.14	7.8	NG	-
3S	4.23	8.0	NG	-
4S	9.75	7.9	NG	-
5S	11.31	7.7	NG	-
6S	17.86	7.8	NG	-
M1	8.95	7.8	NG	-
M2	9.75	7.9	NG	-
M3	7.25	7.9	NG	-
M4	8.76	8.0	NG	-

(a) means of two experiments, each carried out in three replicates.

(°) NG no growth

Table 5 - Morphological and physiological characteristics of the isolated strains

Characteristic	n° of positive strains
Glucose as sole C source	4
Biotine requirement	0
PHB in semisolid malate medium	18
Gas from nitrite	17
Catalase	18
Motility	18
Cell length from 3.5 to 5.0 $\mu\text{m}$	4
Cells shorter than 3.5 $\mu\text{m}$	14

Two groups of isolates were distinguished. One, including the majority of the strains, fitted the description according morphology and physiology of Azospirillum brasilense, while the characteristics of the other group of isolates (strains 1W, 3W, and 5W) suggest their possible placement within the species Azospirillum lipoferum even if they did not fit completely the description as concerns biotine requirement and absence of the typical elongated cells.

All the isolated strains can be temporarily classified as summarized in Table 6.

Table 6 - Proposed systematic position of the isolates

Species	n° of strains
<u>Azospirillum brasilense</u> nir+	13
<u>Azospirillum brasilense</u> nir-	1
<u>Azospirillum lipoferum</u>	4

More detailed work such as DNA base composition and DNA homology studies will be necessary to determine the correct systematic position of these isolates.

The collected data represent the first ecological approach to the occurrence of Azospirillum spp. in the Somalian soils, and confirm the high incidence of these microorganisms in the equatorial zone, as reported by other authors. (17-18).

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